Protein Phosphatase Type 1-Dependent Dephosphorylation of the Retinoblastoma Tumor Suppressor Protein in Ultraviolet-Irradiated Human Skin and Keratinocytes

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This study provides evidence for the involvement of a type 1 protein serine/threonine phosphatase in the ultraviolet radiation-induced dephosphorylation of retinoblastoma tumor suppressor protein in human skin and cultured keratinocytes. The retinoblastoma gene product was localized to the nuclei and nucleoli of keratinocytes, and to the nuclei of basal and spinous layer cells of normal human epidermis. Western blot analysis of the retinoblastoma tumor suppressor protein antigen from keratinocytes and skin established the presence of the hypophosphorylated and hyperphosphorylated forms of retinoblastoma tumor suppressor protein. The exposure of keratinocytes and human skin to 200 J per cm^2 of ultraviolet radiation, resulted in a rapid depletion in hyperphosphorylated retinoblastoma tumor suppressor protein, and the accumulation of growth inhibitory hypophosphorylated retinoblastoma tumor suppressor protein^{105}. In unirradiated and ultraviolet-irradiated keratinocytes retinoblastoma tumor suppressor protein was localized to the spindles of M-phase cells. In contrast, the exposure of keratinocytes to ultraviolet in the presence of 5 mM okadaic acid, resulted in an inhibition of retinoblastoma tumor suppressor protein translocation to the mitotic spindles of M-phase keratinocytes. In addition, the ultraviolet radiation-induced depletion in hyperphosphorylated retinoblastoma tumor suppressor protein, and accumulation of hypophosphorylated retinoblastoma tumor suppressor protein^{105} was inhibited by 5 mM okadaic acid. Okadaic acid (0.5 nM), however, did not affect the ultraviolet radiation-induced dephosphorylation and depletion of hyperphosphorylation of the retinoblastoma tumor suppressor protein. Western blot analysis of ultraviolet-irradiated keratinocytes demonstrated that the hypophosphorylated growth inhibitory 105 kDa form of retinoblastoma tumor suppressor protein coimmunoprecipitated with the 38 kDa catalytic subunit of a type 1 protein serine/threonine phosphatase. ***Key words: human skin/keratinocytes/retinoblastoma tumor suppressor protein/ultraviolet radiation. J Invest Dermatol 115:88–94, 2000***

The skin is the primary target for the carcinogenic effects of sun exposure (Coohill *et al*, 1987). The response of mammalian cells to short wavelength ultraviolet (UV) C between 190 and 290 nm has been extensively studied, and involves the activation of Src tyrosine kinase, Ha-Ras, and Raf-1 kinase (Devary *et al*, 1992; Radler-Pohl *et al*, 1993; Engelberg *et al*, 1994). As a consequence of Raf-1 activation, the mitogen-activated protein kinase pathway is activated, triggering the activation of AP-1 and NF-κB (Devary *et al*, 1993; Hipskind *et al*, 1994). In contrast to short wavelength UVC, the cellular response initiated by UVB between 290 and 320 nm is not well understood. and does not involve activation of the mitogen-activated protein kinase pathway (Roddey *et al*, 1989). UVB, however, has been shown to affect multiple cellular targets that inhibit cell growth by inducing transient delays during the cell cycle (Barker *et al*, 1995; Medrano *et al*, 1995). Transient DNA damage induced cell cycle delays are thought to prevent the propagation of genetic lesions by allowing time for DNA repair or directing the cell into apoptosis (Hartwell, 1992; Hartwell and Kastan, 1994; Strauss *et al*, 1995).

The cell cycle is tightly regulated in mammalian cells by cyclins, cyclin-dependent protein kinases, cyclin-dependent protein kinase inhibitors, tumor suppressor gene products, and other nuclear proteins. The retinoblastoma tumor suppressor (pRB) gene product has a fundamental regulatory role in restricting the inappropriate entry of cells into the cell cycle. (De Caprio *et al*, 1989; Goodrich *et al*, 1991; Weinberg, 1995; Herwig and Strauss, 1997). pRB is a constitutively expressed nuclear phosphoprotein that is phosphorylated and dephosphorylated upon serine and threonine residues in a cell cycle dependent manner (Mihara *et al*, 1989; Chen *et al*, 1989; Ludlow *et al*, 1993; Amellem *et al*, 1996). Hypophosphorylated pRB^{105} is thought to inhibit cell cycle progression by forming protein complexes with three members of the E2F family of transcription factors, which in turn inhibits the expression of E2F regulated S-phase genes (Chellappan *et al*, 1991; Lees *et al*, 1993). Cell cycle progression occurs when hypophosphorylated pRB^{105} is

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Abbreviations: pRB, retinoblastoma tumor suppressor protein; PP1, type 1 serine/threonine protein phosphatase.

phosphorylated on serine and threonine residues by activated cyclin A-Cdk2, D-Cdk4, and cyclin E-Cdk2 complexes (Dowby *et al*, 1993; Ewen *et al*, 1993; Kato *et al*, 1993; Dulic *et al*, 1994; Duronio *et al*, 1996). In the latter stages of mitosis, pRB is rapidly dephosphorylated by a type 1 serine/threonine protein phosphatase (PP1), which results in growth suppressive hypophosphorylated pRB^{105} being translocated to both daughter cells prior to entry into G_1 (Fernandez *et al*, 1992; Alberts *et al*, 1993; Thomas *et al*, 1996; Deirdre *et al*, 1997; Krucher and Ludlow, 1997; Nelson and Ludlow, 1997; Puntoni and Villa-Moruzzi, 1997).

In human fibroblasts, keratinocytes and a number of other cell types, DNA damage induced G_1 growth arrest is mediated by the p53 tumor suppressor protein through the transcriptional activation of $p21^{Waf1/SD1/Cip1}$, which correlates with an inhibition of both cyclins A, D, and E associated kinases, and pRB phosphorylation. (El-Deiry *et al*, 1993; Harper *et al*, 1993; Dulic *et al*, 1994; Medrano *et al*, 1995; Petrocelli *et al*, 1996). Recent studies have demonstrated the presence of a DNA damage dependent, p53-independent growth arrest mechanism, which requires the dephosphorylation of pRB. For example, it has been reported that UV radiation can cause a shift of pRB to its growth inhibitory hypophosphorylated form, in cell lines expressing mutant or inactivated p53. For example, HL-60 human promyelocytic leukemia and U-937 human monocytic leukemia cell lines, which do not contain functional p53, require the dephosphorylation of pRB prior to growth arrest (Michieli *et al*, 1994; Haapajarvi *et al*, 1995). The mechanism by which pRB is dephosphorylated in these p53-deficient cell lines has been reported to involve a PP1 (Dou *et al*, 1995). We have investigated the effect of UV radiation upon pRB expression in human skin and cultured keratinocytes. We report that UV radiation can cause the rapid depletion of hyperphosphorylated pRB, and a 43% increase in hypophosphorylated pRB^{105}, which forms a protein complex with the catalytic subunit of a type1 serine/threonine protein phosphatase.

MATERIALS AND METHODS

Materials Anti-human pRB monoclonal antibody G3–245 recognized an epitope between amino acids 300 and 380 of pRB and was obtained from AMS Biotechnology (Witney, U.K.). Rabbit anti-human pRB polyclonal antibody RB Ab-2 recognized an epitope in the C-terminal domain of human pRB and was obtained from Cambridge Biosciences (Cambridge, U.K.). Anti-human PP1 (FL-18) polyclonal antibody reactive with the catalytic subunits of human PP1, PP2A, PP2B and PPX was obtained from Autogen Bioclear (Calne, U.K.). Dulbecco's modified Eagle's medium and MCDB 153 complete medium was obtained from Gibco, Laboratories (Uxbridge, U.K.). Mouse IgG1 clone DAK-G01 negative control antibody was obtained from DAKO (Ely, U.K.). Okadaic acid, dimethyl sulfoxide, and a 66 kDa bovine albumin prestained molecular weight marker were obtained from the Sigma (Gillingham, U.K.). Biotinylated anti-mouse IgG, biotinylated anti-rabbit IgG, streptavidin horseradish peroxidase complex and Hybond nitrocellulose membranes were supplied by Amersham International PLC (Little Chalfont, U.K.).

Immunohistochemical localization of pRB in human skin Normal human foreskins were obtained from 10 patients attending the day theatre at the University of Wales College of Medicine, Cardiff. The tissues were washed in phosphate-buffered saline (PBS), pH 7.4, cut into 4 mm slices and exposed to 200 J per cm^2 of UV radiation. The UV source consisted of a set of four Westinghouse fluorescent UV tubes (Type FS20) mounted on a curved reflector. The UV source produced UVB with a peak output of 313 nm and small amounts of UVB photons and UVC radiation. Irradiance was routinely measured before each exposure with a UVX digital radiometer (UVP, Cambridgeshire, U.K.) with a UVX-31 detector probe, and peak sensitivity at 310 nm. Irradiated and unirradiated skin slices were incubated in serum-free Dulbecco's modified Eagle's medium for 1, 2, 4, and 8 h at 37°C in an atmosphere of 5% CO_2 in air, washed in PBS and snap frozen in hexane at –70°C and stored in liquid nitrogen. Cryostat sections (4 μm) from irradiated and unirradiated skin slices were fixed in ice-cold acetone, and endogenous peroxidase activity blocked with 0.3% H_2O_2 in methanol for 30 min at 20°C. Sections were blocked with 3% normal sheep serum for 20 min at 20°C and immunolabeled with 2 μg RB Ab-2 polyclonal antibody per ml or 2 mg G3–245 monoclonal antibody per ml in 0.6% bovine serum albumin for 1 h at 37°C. The sections were incubated for 30 min at 20°C with anti-rabbit immunoglobulin diluted 1:50 in PBS. Washed sections were incubated for 15 min at 20°C with biotinylated streptavidin horseradish peroxidase complex (1:125). After extensive washing the primary antibody was visualized using 0.05% diaminobenzidine and 0.003% H_2O_2 in PBS for 5 min and counterstained with eosin and methyl green. Control slides were similarly treated either with the omission of the primary antibody or by incubation with 2 mg IgG1 negative control antibody per ml.

Immunocytochemical localization of pRB in human keratinocytes Keratinocyte suspensions were prepared from human foreskins by incubation for 1 h at 37°C in DSS containing 0.25% trypsin at pH 7.2. Keratinocyte suspensions were plated on to a mitomycin-treated 3T3 fibroblast feeder layer and grown at 37°C in a 3:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 containing 5% fetal bovine serum, 4 mg hydrocortisone per ml, 5 mg insulin per ml, 10 ng cholera toxin per ml, 5 mg transferrin per ml, 10 ng epidermal growth factor per ml and 2×10^{-10} M triiodothyronine. Keratinocytes were subcultured into dishes containing sterile coverslips and incubated at 37°C in MCDB 153 complete medium in a humidified atmosphere of 5% CO_2 in air as previously described (Edwards *et al*, 1990). The medium was removed and the cells exposed to 200 J UV radiation per cm^2 and incubated in fresh medium for up to 8 h at 37°C in the presence and absence of 0.5 nM or 5 mM okadaic acid. The cells were washed in PBS, fixed in methanol at –20°C for 20 min followed by acetone at 4°C for 2 min. The primary polyclonal antibody RB Ab-2 (2 mg per ml), G3–245 monoclonal antibody (2 mg per ml), and IgG1 negative control antibody (2 mg per ml) were diluted in 0.6% bovine serum albumin in PBS and added to separate coverslips for 1 h at 37°C, and the primary antibody visualized as described above. Short-term cell viability was measured by the exclusion of the vital dye Trypan Blue.

Immunoprecipitation and western blot analysis Irradiated and unirradiated human skin slices and keratinocytes were washed in ice-cold PBS and lyzed in buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM ethylenediamine tetraacetic acid, 2 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 0.5% sodium deoxycholate, 0.3%, 0.2% Triton X-100, NP-40, 0.1 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride, 25 μg aprotinin per ml, and 5 mg leupeptin per ml for 30 min at 4°C. The lysates were clarified by centrifugation at $14{,}000 \times g$ and the supernatants precleared by incubating with 100 ml of a slurry of protein-G agarose in PBS (1:1) for 60 min at 4°C. The protein-G agarose was removed by centrifugation and protein estimation performed by BioRad assay (Bio-Rad, Hemel Hempstead, U.K.). Precleared lysates containing 500 mg of protein were incubated for 12 h at 4°C in the presence of 3 mg per ml mouse IgG1 negative control antibody or 3 μg per ml of anti-human pRB monoclonal antibody G3–245. Protein–antibody complexes were precipitated with rabbit anti-mouse IgG conjugated to protein A-Sepharose 4B beads for 1 h at 4°C. Beads were washed in a buffer containing 25 mM Tris–HCl, pH 7.4, 50 mM NaCl, 0.5% sodium deoxycholate, 0.2% Nonidet p-40, and 1 mM phenylmethylsulfonyl fluoride. Equal volumes of washed beads were resuspended in sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer and the immunoprecipitated proteins separated on a 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to Hybond nitrocellulose membranes as previously described (Laemmli, 1970). Nonspecific binding was blocked using 5% powdered milk in PBS for 12 h at 4°C. The membranes were immunolabeled with 2 mg RB-Ab2 polyclonal antibody per ml, G3–245 anti-human pRB monoclonal antibody and 2 μg anti-human PP1 monoclonal antibody per ml in Tris-buffered saline buffer containing 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 5% bovine serum albumin for 2 h at 37°C. Membranes were incubated for 30 min at 20°C with alkaline phosphatase-conjugated goat anti-rabbit antibody (1:7500). The membranes were washed in Tris-buffered saline buffer containing 20 mM Tris–HCl, pH 8.0, and 150 mM NaCl and developed with 5′-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Towbin *et al*, 1979). Even the loading of samples to polyacrylamide gels was verified by staining part of the gel with Coomassie Brilliant Blue. Polypeptide peak areas were measured by computerized densitometry using an LKB Ultrascan laser densitometry (LKB Pharmacia, Uppsala, Sweden). Polypeptide peak areas (AU × mm^2) were normalized against the peak area of a standard polypeptide with a molecular weight of 66 kDa (albumin).

RESULTS

Expression of pRB in human skin **Figure 1**(*a*) illustrates the typical nuclear pRB immunoreactivity of basal and spinous layer

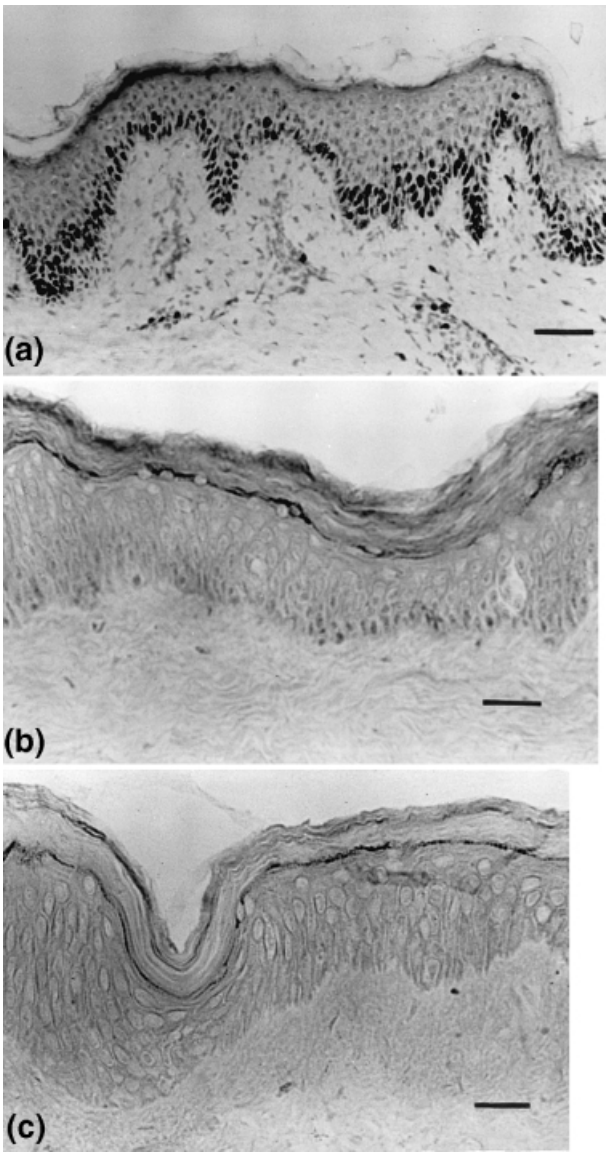

Figure 1. Immunohistochemical localization of pRB in human skin. Skin slices were exposed to 200 J per cm^2 of UV and allowed to recover for up to 8 h at 37°C in growth medium. Unirradiated skin (*a*), showing anti-RB-Ab2 polyclonal antibody staining in the nuclei of basal and spinous layer cells of the epidermis and in the cellular compartment of the dermis. In UV-irradiated skin (*b*), there appears to be a decrease in the nuclear anti-pRB reactivity of the basal and spinous layer cells of the epidermis, and in the cellular compartment of the dermis 4 h after the initial exposure. The absence of nonspecific immunoreactivity in unirradiated skin probed with the IgG1 negative control antibody. *Scale bar*: 250 mm.

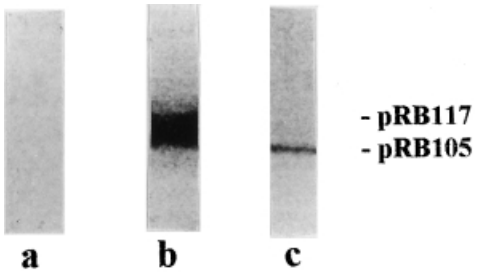

Figure 2. Effect of UV radiation upon the phosphorylation state of pRB in human skin. Unirradiated and irradiated skin slices were incubated in growth medium for 4 h and the polypeptides immunoprecipitated from cell lysates with the G3–245 anti-human pRB monoclonal antibody or IgG1 negative control antibody. Polypeptides were separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the nitrocellulose membranes stained with RB-Ab2 anti-human pRB polyclonal antibody. Human skin (*a*), immunoprecipitated with the IgG1-negative control antibody showing the absence of nonspecific immunoreactivity. Unirradiated skin (*b*), showing the presence of growth inhibitory hypophosphorylated pRB^{105} and hyperphosphorylated forms of pRB. Irradiated skin (*c*), showing the presence of growth inhibitory hypophosphorylated pRB^{105}.

cells of normal unirradiated human epidermis and cellular compartment of the dermis, when probed with the RB Ab2 anti-human pRB polyclonal antibody. Skin slices were also exposed to 200 J per cm^2 of UV radiation and allowed to recover for 1, 2, 4 and 8 h at 37°C. A depletion in anti-pRB reactivity was observed in the nuclei of basal and spinous layer cells 4 h after the initial radiation exposure (**Fig 1*b***). No further depletion in anti-pRB reactivity was observed in the nuclei of basal and spinous layer cells for up to 8 h after the initial exposure. Nonspecific immunoreactivity was not detected in sections from unirradiated or irradiated skin (**Fig 1*c***). Similar pRB immunoreactivity was observed in unirradiated and irradiated skin when the sections were immunostained with the G3–245 anti-human pRB monoclonal antibody (data not shown). The pRB antigen in unirradiated and irradiated skin was characterized by western blot analysis. **Figure 2**(***a***) shows the absence of nonspecific immunoreactivity in skin slices, which had been probed with an IgG1-negative control antibody. **Figure 2**(***b***) illustrates the presence of growth inhibitory hypophosphorylated pRB^{105} and all hyperphosphorylated forms of pRB in unirradiated skin. The exposure of skin slices, however, to 200 J per cm^2 of UV radiation, followed by a postirradiation recovery for 4 h, resulted in the depletion of hyperphosphorylated pRB, and an increase in the amount of growth inhibitory hypophosphorylated pRB^{105} (**Fig 2*c***). Alkaline phosphatase treatment of unirradiated skin lysates caused a decrease in hyperphosphorylated pRB, and a simultaneous increase in the amount of hypophosphorylated pRB^{105}.

Expression of pRB in human keratinocytes **Figure 3** illustrates the anti-pRB reactivity of asynchronous unirradiated keratinocytes when probed with the RB Ab2 anti-human pRB polyclonal antibody. The anti-pRB reactivity of unirradiated and irradiated (200 J per cm^2) keratinocytes was granular and localized to the chromatin and nucleoli of nondividing cells (**Fig 3*a*, *b***). Four hours after the initial UV exposure, however, there appeared to be a decrease in the nuclear and nucleolar pRB immunoreactivity of irradiated keratinocytes (**Fig 3*b***).

Okadaic acid inhibition of PP1 activity in human keratinocytes Protein phosphatase inhibition studies were performed to investigate the UV radiation induced modulation of pRB expression in cultured keratinocytes. It has been reported that the dephosphorylation of pRB^{117} to pRB^{105} involves serine/threonine protein phosphatases (Alberts *et al*, 1993; Durfee *et al*, 1993; Deirdre *et al*, 1997; Krucher and Ludlow, 1997). The PP1 holoenzyme can be distinguished from PP2A and other serine/threonone protein phosphatases by the inhibitory effect of okadaic acid. For example, PP2A has been reported to be inhibited by 0.1–1.0 nM okadaic acid, and PP1 is inhibited by okadaic acid concentrations 100 times higher than that required for the inhibition of PP2A (Higjiki and Suganuma, 1993; Shenolikar, 1994). The effect of okadaic acid upon the UV irradiation-induced depletion and dephosphorylation of pRB was investigated by immunocytochemistry using the RB Ab2 polyclonal antibody. Keratinocytes were exposed to 200 J per cm^2 of UV radiation and incubated for 4 h at 37°C in the presence and absence of 0.5 nM or 5 mM okadaic acid. We previously reported that during M-phase pRB is dephosphorylated and translocated from chromatin to the mitotic spindles of human keratinocytes (Thomas *et al*, 1996). The

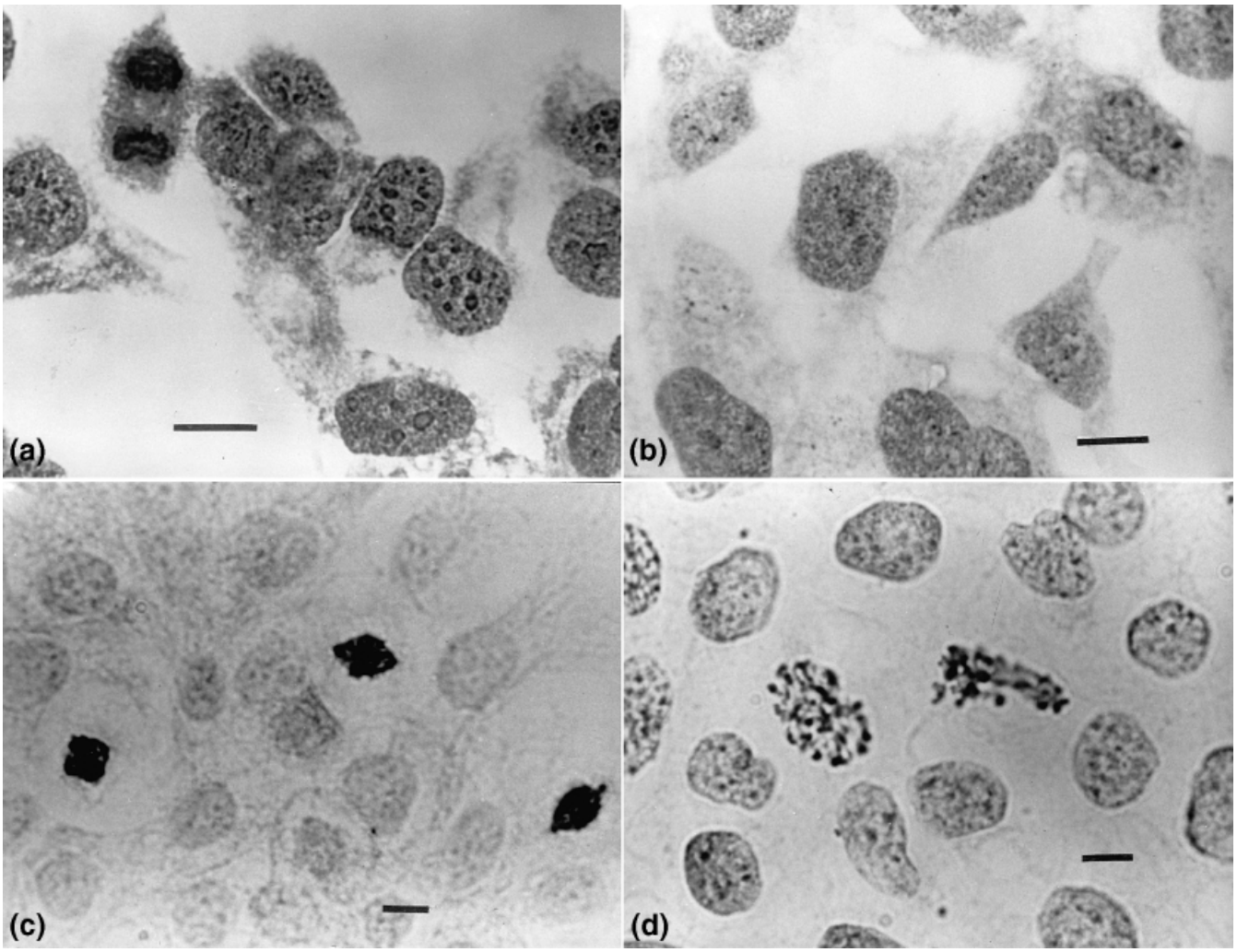

Figure 3. Effect of UV and okadaic acid upon pRB expression in human keratinocytes. Unirradiated and irradiated asynchronous keratinocytes were incubated for 4 h in the presence of 0.5 nM or 5 mM okadaic acid and the cells immunolabeled with RB Ab2. Unirradiated keratinocytes (*a*), showing granular anti-pRB staining in the nuclei and nucleoli. Irradiated keratinocytes (*b*), showing a decrease in nuclear anti-pRB staining. Irradiated and 0.5 nM okadaic acid-treated keratinocytes (*c*), showing the localization of anti-pRB staining to the mitotic spindles. Irradiated and 5 mM okadaic acid-treated keratinocytes (*d*), showing the localization of pRB to the condensed chromatin of M-phase cells. *Scale bar*: 5 mm.

exposure of M-phase keratinocytes to 200 J per cm^2 of UV radiation in the presence of 0.5 nM okadaic acid did not prevent the localization of pRB to the mitotic spindles (**Fig 3*c***). The translocation of pRB to the mitotic spindles of irradiated and 5 mM treated M-phase keratinocytes, however, was inhibited and the anti-pRB reactivity was localized to condensed chromatin (**Fig 3*d***). This suggests that a specific serine/threonine type 1 protein phosphatase is responsible for the dephosphorylation of pRB during M-phase. The effect of okadaic acid upon the UV-induced depletion and dephosphorylation of pRB was investigated by western blot analysis. **Figure 4**(***a***) shows that all forms of pRB were present in asynchronous unirradiated keratinocytes, and **Fig 4**(***b***) shows the absence of nonspecific immunoreactivity in keratinocyte cell lysates when immunoprecipitated with the IgG1 negative control antibody. The exposure of keratinocytes to UV in the presence of 0.5 nM okadaic acid, resulted in the isolation of only growth inhibitory hypophosphorylated pRB^{105} (**Fig 4*c***). The exposure of keratinocytes to UV in the presence of 5 mM okadaic acid, however, resulted in the isolation of all forms of pRB, and the radiation-induced dephosphorylation and the depletion of hyperphosphorylated pRB were inhibited (**Fig 4*d***).

The pRB immunoprecipitates from six unirradiated and irradiated keratinocyte cell lysates were quantitated by scanning densitometry. **Figure 5**(***b***) shows that irradiated and 0.5 nM okadaic acid-treated keratinocytes contained 43% more hypophosphorylated growth inhibitory pRB^{105} when compared with unirradiated controls. The depletion of hyperphosphorylated pRB in irradiated keratinocytes was not the result of general protein degradation because protein loading was equal in control and irradiated cell lysates. **Figure 5**(***c***) also shows that 5 mM okadaic acid inhibited the radiation-induced dephosphorylation and depletion of hyperphosphorylated pRB.

Co-immunoprecipitation of pRB with PP1 The radiation induced dephosphorylation of pRB in cultured keratinocytes was investigated further by western blot analysis. Irradiated keratinocytes were immunoprecipitated with the IgG1-negative control antibody, G3–245 anti-human pRB monoclonal antibody and anti-PP1 monoclonal antibody. The polypeptides were separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes and stained with the RB Ab2 anti-pRB polyclonal antibody and anti-human PP1 monoclonal antibody. **Figure 6**(***a***, ***e***) show the position of a 66 kDa polypeptide molecular weight marker. **Figure 6**(***b***, ***f***) illustrates the absence of nonspecific immunoreactivity when irradiated keratinocyte cell lysates were immunoprecipitated with

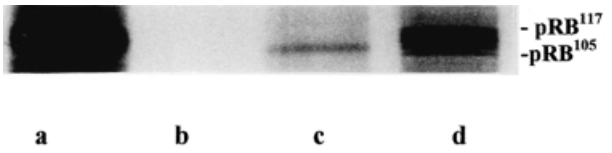

Figure 4. Effect of UV and okadaic acid upon the phosphorylation state of pRB in human keratinocytes. Unirradiated and irradiated keratinocytes were incubated for 4 h in the absence and presence of 0.5 nM and 5 mM okadaic acid. The polypeptides from cell lysates were immunoprecipitated with the G3–245 anti-pRB monoclonal antibody or IgG1 negative control antibody and the membranes probed with the RB-Ab2 anti-pRB polyclonal antibody. Unirradiated keratinocytes (*a*), showing the presence of all forms of pRB. Control keratinocytes (*b*), immunoprecipitated with the IgG1-negative control antibody. Irradiated and 0.5 nM okadaic acid-treated keratinocytes (*c*), showing the presence of the growth inhibitory pRB^{105}. Irradiated and 5 mM okadaic acid-treated keratinocytes (*d*) showing the presence of growth inhibitory pRB^{105} and all hyperphosphorylated forms of pRB.

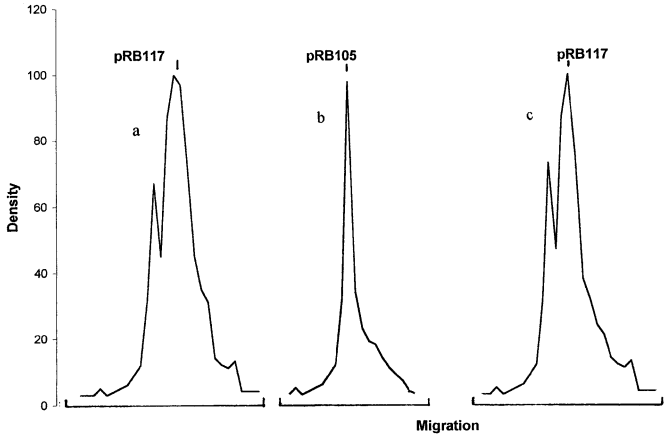

Figure 5. Quantitation of pRB expression in human keratinocytes. The relative proportion of the hypophosphorylated pRB^{105} and hyperphosphorylated forms of pRB in irradiated and unirradiated keratinocytes were determined by scanning densitometry. Unirradiated keratinocytes (*a*), showing the presence of all forms of pRB. Irradiated and 0.5 nM okadaic acid-treated keratinocytes (*b*), showing the presence of growth inhibitory pRB^{105}. Irradiated and 5 mM okadaic acid-treated keratinocytes (*c*), showing the presence of all forms of pRB.

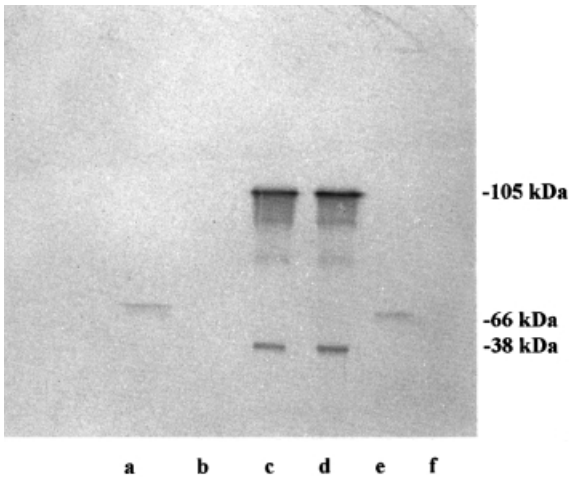

Figure 6. Co-immunoprecipitation of pRB with PP1 from irradiated human keratinocytes. Irradiated keratinocytes were allowed to recover for 4 h at 37°C, and the cell lysates immunoprecipitated with the IgG1-negative control antibody or G3–245 anti-human pRB monoclonal antibody. The membranes were probed with the RB-Ab2 polyclonal antibody and anti-human PP1 monoclonal antibody. A 66 kDa bovine albumin prestained polypeptide molecular weight marker (*a*, *e*). Keratinocytes immunoprecipitated with the IgG1-negative control antibody showing the absence of nonspecific immunoreactivity (*b*, *f*). Polypeptides immunoprecipitated from irradiated keratinocytes with the anti-PP1 monoclonal antibody (*c*), showing the presence of growth inhibitory pRB^{105} and a 38 kDa polypeptide with anti-PP1 immunoreactivity. Irradiated keratinocytes (*d*), immunoprecipitated with the anti-pRB monoclonal antibody G3–245 and stained with the RB-Ab2 polyclonal antibody and anti-PP1 monoclonal antibody.

the IgG1 negative control antibody. Immunoprecipitation of PP1 from irradiated keratinocytes with the anti-PP1 monoclonal antibody resulted in the coprecipitation of pRB^{105} with a 38 kDa polypeptide with anti-PP1 reactivity (**Fig 6*c***). **Figure 6(*d*)** shows the presence of anti-pRB reactivity with an apparent polypeptide molecular weight of 105 kDa and a 38 kDa polypeptide with anti-PP1 reactivity in irradiated keratinocyte immunoprecipitated with the G3–245 anti-pRB monoclonal antibody.

DISCUSSION

One explanation for the UV radiation-induced decrease in the nuclear anti-pRB staining of human skin and cultured keratinocytes is the report that the C-terminal region recognized by the RB Ab2 polyclonal antibody undergoes specific cell cycle stage-dependent phosphorylations, which induce conformational changes in pRB and inhibit antibody recognition and binding. This explanation is unlikely as similar anti-pRB staining was observed in irradiated skin and keratinocytes when probed with the G3–245 monoclonal antibody, which recognized an epitope between amino acids 300–380 of pRB and does not undergo cell cycle dependent phosphorylation (Hu, 1991; Haapajarvi *et al*, 1995). Whereas the peak emission of the UVB source used in the study was 313 nm, the presence of UVC and very short wavelength UVB photons may, as yet, have unidentified implications with respect to the interpretation of our data. We favor the view, however, that UVB radiation can rapidly cause hyperphosphorylated pRB depletion in human keratinocytes. In support of this observation is the report which shows that, in human melanocytes, the phosphorylation of pRB was inhibited after a monocytostatic exposure to UVB (Medrano *et al*, 1995). The mechanism by which UVB radiation inhibited the phosphorylation of pRB is unknown. It has been reported that in human melanocytes and MM96L human melanoma cells, UVB exposure did not cause the loss of pRB mRNA and it did not affect pRB protein stability; however, it specifically inhibited the translation of pRB for up to 24 h after the initial exposure (Pedley *et al*, 1996). These observations provided indirect evidence that the inhibition of pRB phosphorylation in UVB-irradiated melanocytes contributes to malignancy by continuing cell division before DNA repair is complete (Medrano *et al*, 1995).

Keratinocytes, however, may be less sensitive to the effects of UVB radiation when compared with melanocytes, as the radiation-induced decline in hyperphosphorylated pRB was accompanied by the rapid synthesis of growth inhibitory hypophosphorylated pRB^{105}. Coprecipitation studies also showed that hypophosphorylated pRB^{105} forms a protein complex with the 38 kDa catalytic subunit of PP1 in irradiated keratinocytes. During G_1 and S phase, PP1 is cytoplasmic and accumulates in the nucleus during M-phase, where it becomes associated with condensed chromatin. The cell cycle dependent changes in PP1 location and activity are consistent with the view that it has a cell cycle regulatory role, which promotes the dephosphorylation of pRB and other nuclear phosphoproteins (Pallen *et al*, 1992; Deirdre *et al*, 1997; Krucher and Ludlow, 1997; Nelson and Ludlow, 1997; Krtolica *et al*, 1998).

Our data suggest that the PP1-dependent dephosphorylation of pRB may function in DNA damage-induced p53-independent

growth arrest. This is supported by a study that the UVB irradiation of human and mouse fibroblasts results in the rapid dephosphorylation of pRB and G_1 growth arrest, which occurs independently of p53 (Haapajarvi *et al*, 1995). Secondly, when two p53 null human leukemic cell lines were treated with a variety of anticancer drugs, pRB became hypophosphorylated and this was accompanied by G_1 growth arrest. PP1 was implicated in this process, as the addition of the serine/threonine protein phosphatase inhibitor okadaic acid prevented the drug-induced dephosphorylation of pRB and G_1 growth arrest (Dou *et al*, 1995). It has also been reported that the UVB irradiation of melanocytes and murine keratinocytes results in G_1 growth arrest and prolonged inhibition of pRB phosphorylation, which is associated with the long-term expression of $p21^{WAF1//SD1/Cip1}$ without p53 (Medrano *et al*, 1995; Liu *et al*, 1999). Therefore, human keratinocytes may contain a UVB radiation-induced p53-independent growth arrest mechanism, which could involve the induction of $p21^{WAF1/SD1/Cip1}$ and the formation of growth inhibitory pRB^{105}–PP1 protein complexes.

Recent studies have focused on the role of hypophosphorylated pRB in the promotion of p53-independent apoptosis. It has been proposed that an early stage of DNA damage-induced p53-independent apoptosis requires the dephosphorylation of pRB into its hypophosphorylated active form. In HL60 human promyelocytic leukemia and U-937 human monocytic leukemia cell lines, it has been shown that the dephosphorylation of pRB is an early event in the induction of apoptosis, whereas upon the rescue of cells from apoptosis the hyperphosphorylated form of pRB predominated (Dou *et al*, 1995). It has been proposed that the commitment of cells to apoptosis involves the dephosphorylation of pRB by a serine/threonine protein phosphatase and the execution of apoptosis involves the cleavage of newly formed hypophosphorylated pRB by a DNA damage activated interleukin-1b-converting enzyme (Haas-Kogan *et al*, 1995; An and Dou, 1996). Studies are now in progress to determine the relationship between the long-term expression of $p21^{WAF1/SD1/Cip1}$ and PP1–pRB^{105} protein complexes in UVB radiation-induced growth arrest and apoptosis.

REFERENCES

Alberts AS, Thorburn AM, Shenolikar S, Mumby MC, Feramisco JR: Regulation of cell cycle progression and the nuclear affinity of the retinoblastoma protein by protein phosphatases. *Proc Natl Acad Sci USA* 90:388–392, 1993

Amellem O, Stokke T, Sandvik JA, Pettersen EO: The retinoblastoma gene product is reversibly dephosphorylated and bound in the nucleus of S and G_2 phases during hypoxic stress. *Exp Cell Res* 227:106–115, 1996

An B, Dou QP: Cleavage of retinoblastoma protein during apoptosis: an interleukin 1 beta-converting enzyme-like protease as candidate. *Cancer Res* 56:428–442, 1996

Barker D, Dixon K, Medrano EE, *et al*: Comparison of the responses of human melanocytes with different melanin contents to UVB irradiation. *Cancer Res* 55:4041–4046, 1995

Chellappan SP, Hiebert S, Mudryl M, Horowitz JM, Nevins JR: The E2F transcription factor is a cellular target for the RB protein. *Cell* 65:1053–1061, 1991

Chen P-L, Scully P, Shew J-Y, Mudryj M, Horowitz JM, Nevins JR: Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell* 58:1193–1198, 1989

Coohill T, Peak M, Peak J: The effects of ultraviolet wavelengths of radiation present in sunlight on human cells in vitro. *Photochem Photobiol* 46:1043–1050, 1987

De Caprio JA, Ludlow JW, Lynch D: The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. *Cell* 58:1085–1095, 1989

Deirdre AN, Krucher NA, Ludlow JW: High molecular weight protein phosphatase type 1 dephosphorylates the retinoblastoma protein. *J Biol Chem* 272:4528–4535, 1997

Devary Y, Gottlieb RA, Seal T, Karin M: The mammalian ultraviolet response is triggered by activation of Src tyrosine kinase. *Cell* 71:1081–1091, 1992

Devary Y, Rosette C, DiDonato JA, Karin M: NF-κB activation by ultraviolet light is not independent on a nuclear signal. *Science* 261:1442–1445, 1993

Dou QP, An B, Will PL: Induction of the retinoblastoma phosphatase activity by anticancer drugs accompanies p53-independent G_1 arrest and apoptosis. *Proc Natl Acad Sci USA* 92:9019–0923, 1995

Dowby SF, Hinds PW, Louie K: Physical interactions of the retinoblastoma protein with human D cyclins. *Cell* 73:499–511, 1993

Dulic V, Kaufmann WK, Wilson SJ, *et al*: p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation induced G1 arrest. *Cell* 76:1013–1023, 1994

Durfee T, Becherer K, Chen P-L: The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev* 7:555–569, 1993

Duronio RJ, Brook A, Dyson N, O'Farrell PH: E2F induced S phase requires cyclin E. *Genes Dev* 10:2505–2513, 1996

Edwards MJ, Dykes PJ, O'Donovan MR, Merrett VR, Morgan HE, Marks R: Induction of heat shock proteins as a measure of chemical cytotoxicity. *Toxicology In Vitro* 4:270–276, 1990

El-Deiry WS, Tokino T, Velculescu VE: WAF1, a potential mediator of p53 tumour suppression. *Cell* 75:817–825, 1993

Engelberg D, Klein C, Martinetto H, Struhl K, Karin M: The UV response involving the Ras signaling pathway and AP-1 transcription factors is conserved between yeast and mammals. *Cell* 77:381–390, 1994

Ewen ME, Sluss HK, Sheer CJ, Matsushime H, Kato J, Livingstone DM: Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell* 73:487–497, 1993

Fernandez A, Brautigan DL, Lamb NJC: Protein phosphatase type 1 in mammalian cell mitosis: Chromosomal localization and involvement in mitotic exit. *J Cell Biol* 116:1421–1430, 1992

Goodrich DW, Wang NP, Qian YW, Lee EY, Lee WH: The retinoblastoma gene product regulates progression through the G_1 phase of the cell cycle. *Cell* 67:293–302, 1991

Haapajarvi T, Kivinen L, Pitkanen K, Laiho M: Cell cycle dependent effects of UV-radiation on p53 expression and retinoblastoma protein phosphorylation. *Oncogene* 11:151–159, 1995

Haas-Kogan DA, Kogan SC, Levi D, Dazin P, T'Ang A, Fung YK, Israel MA: Inhibition of apoptosis by the retinoblastoma product. *EMBO J* 14:461–472, 1995

Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ: The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G_1 cyclin dependent kinases. *Cell* 75:805–816, 1993

Hartwell L: Defects in cell cycle checkpoint may be responsible for the genomic instability of cancer cells. *Cell* 71:543–546, 1992

Hartwell LH, Kastan MB: Cell cycle control and cancer. *Science* 266:1821–1828, 1994

Herwig S, Strauss M: The retinoblastoma protein: a master regulator of cell cycle, differentiation and apoptosis. *Eur J Biochem* 246:581–601, 1997

Higjiki H, Suganuma M: Tumour promotion by inhibition of protein phosphatase 1 and 2A the okadaic acid class of compounds. *Adv Cancer Res* 61:143–194, 1993

Hipskind RA, Baccarino M, Nordheim A: Transient activation of RAF-1, MEK and ERK2 coincides kinetically with ternary complex factor phosphorylation and immediate early gene promoter activity *in vivo*. *Mol Cell Biol* 14:6219–6231, 1994

Hu Q, Bautista C, Edwards GM, Defeo-Jones D, Jones RE, Harlow E: Antibodies specific for the human retinoblastoma protein identify a family of related polypeptides. *Mol Cell Biol* 11:5792–5799, 1991

Kato J, Matsushime H, Hiebert SW, Ewen ME, Sherr CJ: Direct binding of cyclin D to the retinoblastoma gene product (pRB) and pRB phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev* 7:331–342, 1993

Krtolica A, Krucher NA, Ludlow JW: Hypoxia-induced pRB hypophosphorylation results from downregulation of CDK and upregulation of PP1 activities. *Oncogene* 17:2295–2304, 1998

Krucher NDA, Ludlow JW: High molecular weight protein phosphatase type 1 dephosphorylates the retinoblastoma protein. *J Biol Chem* 272:4528–4535, 1997

Laemmli UK: Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970

Lees JA, Saito M, Vidal M: The retinoblastoma protein binds to a family of E2F transcription factors. *Mol Cell Biol* 13:7813–7825, 1993

Liu M, Wikonkal NM, Brash DE: UV induces $p21^{WAF1/CIP1}$ protein in keratinocytes without p53. *J Invest Dermatol* 113:283–284, 1999

Ludlow JW, Glendening CL, Livingstone DM, DeCaprio JA: Specific enzymatic dephosphorylation of the retinoblastoma protein. *Mol Cell Biol* 13:367–372, 1993

Medrano EE, Im S, Yang F, Abdel-Malek ZA: Ultraviolet B light induces G_1 arrest in human melanocytes by prolonged inhibition of retinoblastoma protein phosphorylation associated with long term expression of the $p21^{Waf-1/SDI-1/Cip-1}$ protein. *Cancer Res* 55:4047–4052, 1995

Michieli P, Chedid M, Lin D, Pierce JH, Mercer WE, Givol D: Induction of WAF1/CIP1 by a p53-independent pathway. *Cancer Res* 54:3391–3395, 1994

Mihara K, Cao XR, Yen A: Cell cycle-dependent regulation of phosphorylation of human retinoblastoma gene product. *Science* 246:1300–1303, 1989

Nelson DA, Ludlow JW: Characterisation of the mitotic phase pRB-directed protein phosphatase activity. *Oncogene* 14:2407–2415, 1997

Pallen CJ, Tan YH, Guy G: Protein phosphatases in cell signalling. *Curr Opin Cell Biol* 4:1000–1007, 1992

Pedley J, Ablett EM, Pettit A, Meyer J, Dunn IS, Sturn RA, Parsons PG: Inhibition of retinoblastoma protein translation by UVB in human melanocytic cells and reduced cell cycle arrest following repeated irradiation. *Oncogene* 13:1335–1342, 1996

Petrocelli T, Poon R, Drucker DJ, Slingerland JM, Rosen CF: UVB radiation induces $p21^{Cip1/WAF1}$ and mediates G_1 and S phase checkpoints. *Oncogene* 1:1387–1396, 1996

Puntoni F, Villa-Moruzzi E: Association of protein phosphatase-1delta with the retinoblastoma protein and reversible phosphatase activation in mitotic HeLa cells and in cells released from mitosis. *Biochem Biophys Res Comm* 235:704–708, 1997

Radler-Pohl A, Sachsenmaier C, Gebel S: UV-induced activation of AP-1 involves

obligatory extranuclear steps including Raf-kinase. *EMBO J* 12:1005–1012, 1993

Roddey PK, Garmyn M, Park H-Y, Bhawan J, Gilchrist BA: Ultraviolet irradiation induces c-fos but not c-Ha-Ras protoncogene expression in human epidermis. *J Invest Dermatol* 102:296–299, 1989

Shenolikar S: Protein serine/threonine phosphatases—new avenues for cell regulation. *Annu Rev Cell Biol* 10:55–86, 1994

Strauss M, Lukas J, Bartek J: Unrestricted cell cycling and cancer. *Nat Med* 1:1245–1246, 1995

Thomas RC, Edwards MJ, Marks R: Translocation of the retinoblastoma gene product during mitosis. *Exp Cell Res* 223:227–232, 1996

Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–4354, 1979

Weinberg RA: The retinoblastoma protein and cell cycle control. *Cell* 81:323–330, 1995